

Comparative efficacies of three acid-fast staining techniques under field conditions for *Mycobacterium tuberculosis* in the Indian context

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Citation

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Abstract

Tuberculosis is a most important human disease in its global prevalence. It is caused by tubercle bacilli. Among the various methods for diagnosis of pulmonary tuberculosis, bacteriological methods are more important. Standard Ziehl-Neelsen method of staining is most reliable for this. But due to some operational disadvantages in the Ziehl-Neelsen method, many other methods were also discovered. In this research work two such techniques that is Modified Ziehl-Neelsen method where a boiling water bath is used as a source of heating and cold staining method where heating is completely eliminated and instead of which duration of exposure to carbol-fuchsin stain prolonged to 10 minutes were compared with conventional Ziehl-Neelsen method. For these 145 sputum samples having the symptoms of tuberculosis were collected and each of these samples were stained by three above mentioned methods. The results were analyzed by Mc Nemar's chi-square test and kappa statistics. It was observed that Cold-staining method is more appropriate than that of the Modified Ziehl-Neelsen method. Additionally due to some advantages in cold staining method it is more reliable and safer than that of the standard Ziehl-Neelsen method in field conditions.

INTRODUCTION

Tuberculosis is a communicable disease due to infection by tubercle bacilli. Every year approximately 8.8 million new cases of tuberculosis are detected^[1]. About 20% of the world's population of tuberculosis patients resides in India^[2]. According to WHO expert committee, a case of tuberculosis refers to a person with 'bacteriologically confirmed disease' that is a person who expectorates tubercle bacilli in his sputum^[3]. As per as report given by WHO, there were 8.8 million new cases of tuberculosis globally in 2003 with an expected 1% increase annually and 1.7 million deaths worldwide^[4,5]. This disease is also associated with human immunodeficiency virus infection (HIV)^[5]. About 9% of tuberculosis infected patients are HIV positive. The rate of infection and risk is higher in Asian countries including India with a 35 per cent of all cases of worldwide alone in India and China^[5]. Correct and rapid diagnosis of the disease is very important to control the growing epidemics^[4,6]. Laboratory diagnosis of pulmonary tuberculosis is mainly dependent on the demonstration of acid-fast bacilli in sputum by direct microscopy or by culture methods^[7]. Smear microscopy being simpler, quicker and

cheaper is widely used for detecting tubercle bacilli^[8] though it lacks sufficient sensitivity^[7]. Culture of mycobacterium is also beneficial for diagnosis of this disease, but the organism is slow growing and the method is laborious as well as expensive. There are some other good methods for diagnosis such as BACTEC radiometric system which need 4 weeks for detection and sensitivity testing and other rapid culture methods including Septi-Chek AFB, Becton-Dickinson and slide culture method^[7]. Recently amplification of mycobacterial DNA using PCR is in practice in developed countries, but not in developing countries^[6]. The methods except direct microscopy are expensive, biohazardous and need special set up for working, this is for that reasons these methods are not universally available^[7]. So, the direct microscopy technique is more reliable if we can improve their deficiencies^[7]. Ziehl-Neelsen's staining is commonly used for detecting tubercle bacilli. This technique requires heating of the smear, while staining heating is usually done either by using spirit lamps or by Bunsen burners. Further, in this technique a high degree of precision is required i.e. overheating may char the smear and under heating may result in pale staining

of the bacilli. Number of modifications has been made in this technique to avoid the heating or use of flame^[9, 10, 11, 12, 13, 14, 15, 16, 17]. In modified ZN method (MZN) instead of heating directly in the flame the slides are placed on water bath and heated by exposing to steam^[18]. In this study, the efficacies of Modified Z-N method (MZN) and cold staining method of Vasanthakumari.R et al., 1986 (CSV)^[15] were compared with that of the standard ZN technique.

MATERIALS AND METHODS

145 sputum samples were collected from patients having the symptoms of pulmonary tuberculosis like cough of more than 15 days with expectoration, chest pain, low grade fever, loss of weight, loss of appetite etc. All this patients attended the out patient Department of Govt Thiruvatteeswaran Hospital for Thoracic Medicine, Chennai.

PREPARATION OF SMEARS

One loopful of the thickest portion of the sputum sample was spread evenly in the centre of a clean glass slide. It was air dried and heat fixed. Three such smears were made from each sputum sample and labeled as A, B and C after writing the specimen number. "A" group of smears were stained by the standard Z-N method; "B" group of smears by the modified Z-N method and "C" group of smears were stained by the cold staining method (CSV).

PREPARATION OF REAGENTS

Carbol fuchsin (1%) was prepared from 10 g of basic fuchsin (Hi-Media) dissolved in 100 ml of methanol (Qualigens) and 50 ml of melted phenol (Qualigens) in a flask maintained at 60°C in a water bath. This solution was made up to 1,000 ml with distilled water and filtered after proper mixing. Sulfuric acid (25%) was prepared from 250 ml of concentrated sulfuric acid (Qualigens) slowly added to 750 ml of distilled water. Methylene blue (0.1%) was prepared from 1 g of methylene blue (Hi-Media) dissolved in 1,000 ml of distilled water. Gabbett's Methylene blue was prepared as described by Vasanthakumari et al^[15] and Gokhale et al^[19].

STAINING

ZN Method-The glass slides were kept in a staining rack with the smear side facing upwards and flooded with Carbol fuchsin (1%) solution. The slides were heated from underneath using a flame until vapors started rising. After 5 minutes the slides were washed gently with running water, excess water was drained off and 25% Sulphuric acid was poured onto the slides and allowed to stand. After 2 to 3

minutes the slides were washed in running water and excess drained off. Methylene blue solution (0.1%) was poured on the slides and allowed to stand. After 1 minute the slides were rinsed in running water, air dried and examined using oil immersion objective (100x).

MZN Method- The procedure was same as standard ZN method except in the heating step a boiling water bath was used in place of direct flame.

Cold staining Method (CSV) - Slides were placed on the staining rack with the smear facing upwards. Smears were flooded with Carbol fuchsin (1%) stain and allowed to stand at room temperature for 10 minutes. After that slides were washed gently in running tap water and Gabbett's Methylene blue was poured on the smear and allowed to stand for 2 minutes. After that the slides were washed gently in running water, air dried and examined using oil immersion objective (100x).

GRADING

The slides were graded as per the recommendations of the International Union against Tuberculosis and Lung Disease^[20].

STATISTICAL ANALYSIS

The association between results obtained by the three methods was measured using the kappa estimator^[21] a proportional measure varying between -1 (negative agreement) and +1 (positive agreement). Assuming a normal distribution of kappa, confidence limits were estimated using the kappa variance^[21]. A confidence interval of kappa that does not include zero suggests an agreement significantly greater than would be expected by chance alone. The association was considered moderate, substantial, or almost perfect if values were estimated at $0.4 \leq \kappa < 0.6$, $0.6 \leq \kappa < 0.8$, or $0.8 \leq \kappa \leq 1.0$, respectively.

The hypothesis that there were no differences among the results of the methods was evaluated statistically by Mc Nemar's Chi-square (χ^2) test. The probability that the observed differences in results were due to chance was calculated using the binomial distribution. P values below 0.05 were considered significant.

RESULTS

Out of 145 specimens that were examined, 77(53.1%) were positive by standard Ziehl-Neelsen method, 66(45.52%) by Modified Ziehl-Neelsen method and 67(46.21%) by Cold

staining method. On the other hand 68 (46.9%), 79(54.48%) and 78(53.79%) were negative by Ziehl-Neelsen method, Modified Ziehl-Neelsen method and Cold staining (CSV) method respectively [Table 1, Fig 1].

Figure 1

Figure 1- Staining results for sputum samples using three acid-fast staining techniques. [ZN= standard Ziehl-Neelsen method, MZN= Modified Ziehl-Neelsen method and CSV= The Cold staining method]. Vertical bars represents SE.

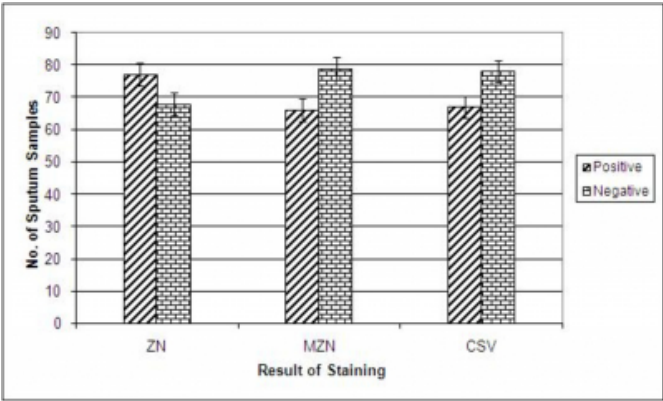


Figure 2

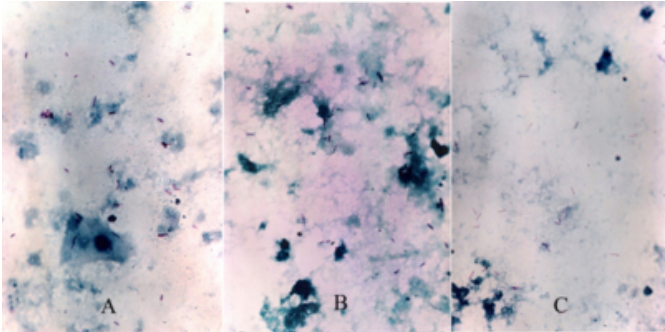
Table 1: Comparison of Modified Ziehl-Neelsen method (MZN) and Cold staining method (CSV) with standard Ziehl-Neelsen method (ZN) results of 145 samples for detection of

| ZN | MZN | | CSV | |
|----------|----------|----------|----------|----------|
| | Positive | Negative | Positive | Negative |
| Positive | 60 | 17 | 65 | 12 |
| Negative | 6 | 62 | 2 | 66 |

It was observed that the bacilli appeared bright pink in all the methods [Fig.2]. But thinner bacilli were observed in Cold staining method than that of the other two methods [Fig.2]. In the Modified Ziehl-Neelsen method, the background was not uniformly stained and was more pinkish [Fig.2].

Figure 3

Figure 2- Common sputum sample stained with (A) the Ziehl-Neelsen (ZN) method, (B) the Modified Ziehl-Neelsen (MZN) method and (C) the Cold staining (CSV) method. (Magnification X 1,000)



Sensitivity, specificity and efficiency of Modified Ziehl-Neelsen method were 77.92%, 91.18% and 84.14% respectively whereas 84.42%, 97.06% and 90.34% were for Cold staining method respectively [Table 2].

Figure 4

Table 2: Statistical evaluation of Modified Ziehl-Neelsen method (MZN) and Cold staining method (CSV) with standard Ziehl-Neelsen method (Z-N) results.

| | Mc Nemar's Chi-square (χ^2) | Kappa (κ) | Sensitivity | Specificity | Efficiency |
|------------|------------------------------------|--------------------|-------------|-------------|------------|
| ZN vs. MZN | 4.35* | 0.68 | 77.92% | 91.18% | 84.14% |
| ZN vs. CSV | 5.79** | 0.81 | 84.42% | 97.06% | 90.34% |

* $P<0.05(3.841)$ and ** $P<0.02(5.412)$ was considered as significant

The differences were statistically significant (Chi-square = 4.35 and 5.79, $P<0.05$) and comparable (kappa = 4.35 and 5.79).

DISCUSSION

The major difficulty in staining tubercle bacilli is due to the presence of an unsaponifiable waxy substance on their surface. Tubercle bacilli are gram positive, aerobic, non-spore, acid-fast, slightly curved or straight non-motile rods [22]. They have the capacity to synthesize large amount of lipids in the form of neutral fats, free fatty acids, phosphatides and waxes [23, 24]. These waxes and the presence of complex three compartmented cell wall are mainly responsible for contributing bacilli the acid-fastness property [22, 23, 25]. Ziehl-Neelsen's staining is commonly used for detecting tubercle bacilli. On staining with carbol-fuchsin followed by heating it enters into the cell [15]. This cationic dye forms a complex with intracellular lipid layer preventing the trapped carbol-fuchsin from being removed and cell-wall components

prevents the entry of decolorizing agent and they appear as red or bright pink colored rods^[22, 24, 26]. But for non acid-fast bacilli the decolorizing agent removes the primary stain and they take the colour of counter stain^[24]. The success of any staining technique depends on the ability of the dye to uniformly penetrate the cell wall through this waxy barrier without affecting the acid-fast character of the organism. In the conventional Ziehl-Neelsen method it is achieved by heating the slides during the staining process. This operation requires a precise heating control and experience on the part of the laboratory technician. Over heating may char the smear and under heating may not be sufficient for the bacilli to take up the stain. Both the conditions lead to false negative. While carrying out the conventional method spirit lamps are used as a source of heating. Very often this method cannot be carried out because of non-availability of rectified spirit. Moreover tough administrative procedures have to be followed while procuring rectified spirit.

To overcome this problems a modified version of Ziehl-Neelsen method is in use where the slides are heated by keeping them on a boiling water bath. Here steam is used as a source of heat. In this study it was found that the quality of staining is not as good as the standard Ziehl-Neelsen method. Using boiling water bath is a risk and cumbersome procedure. Here quantity of staining solutions required is more. More number of slides cannot be stained at a time.

Use of this cold staining procedure has many distinct advantages. A high level of technical skill is not required and so the method is technically simple. Since heating is eliminated, there is no need for rectified spirit and so the procedure is suitable for use even in remote areas and under field conditions. In case of CSV method, fewer reagents are required in compare to MZN methods. Here fixation of smears on slides can be carried out using any available source of dry heat, such as a hot plate, or the closed lid of the boiling sterilizer. In addition, ready made staining solutions are commercially available. Another important advantage of the cold method is that a large number of slides can be stained at a time. Since bacilli appear slender in the cold method it is nearer to natural morphology in the host.

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